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(54) Title: IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNA INHIBITION

(57) Abstract: There are described ways of improving the efficiency of double stranded RNA inhibition as a method of inhibiting gene expression in nematode worms such as *C. elegans*. In particular, the invention relates to the finding that changes in the genetic background of *C. elegans* result in increased sensitivity to double-stranded RNA inhibition.

# IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNA INHIBITION

The present invention is concerned with ways of improving the efficiency of double stranded RNA inhibition as a method of inhibiting gene expression in nematode worms such as *C. elegans*. In particular, the invention relates to the finding that the susceptibility of nematode worms such as *C. elegans* to double stranded RNA inhibition is affected by changes in the genetic background of the worms.

It has recently been described in Nature Vol 391, pp.806-811, February 98, that introducing double stranded RNA into a cell results in potent and specific interference with expression of endogenous genes in the cell, which interference is substantially more effective than providing either RNA strand individually as proposed in antisense technology. This specific reduction of the activity of the gene was also found to occur in the nematode worm Caenorhabditis elegans (*C. elegans*) when the RNA was introduced into the genome or body cavity of the worm.

The present inventors have utilized the double stranded RNA inhibition technique and applied it further to devise novel and inventive methods of (i) assigning functions to genes or DNA fragments which have been sequenced in various projects, such as, for example, the human genome project and which have yet to be accorded a particular function, and (ii) identifying DNA responsible for conferring a particular phenotype. Such methods are described in the applicant's co-pending application number WO 00/01846. Processes for introducing RNA into a living cell, either *in vivo* or *ex vivo*, in order to inhibit expression of a target gene in that cell are

additionally described in WO 99/32619.

Several different experimental approaches can be used to introduce double-stranded RNA into nematode worms in order to achieve RNA interference *in vivo*. One of the most straightforward approaches is simple injection of double-stranded RNA into a body cavity. A more elegant solution is to feed the nematodes on food organisms, generally bacteria, which express a double stranded RNA of the appropriate sequence, corresponding to a region of the target gene.

The present inventors have now determined that the phenomenon of RNA interference in nematodes following ingestion of food organisms capable of expressing double-stranded RNA is dependent both on the nature of the food organism and on the genetic background of the nematodes themselves. These findings may be exploited to provided improved methods of double-stranded RNA inhibition.

Therefore, according to a first aspect of the present invention there is provided a method of inhibiting expression of a target gene in a nematode worm comprising feeding to said nematode worm a food organism which is capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a portion of said target gene following ingestion of the food organism by the nematode, wherein the nematode has a non wild-type genetic background selected to provide increased sensitivity to RNA interference as compared to wild type.

*Caenorhabditis elegans* is the preferred nematode worm for use in the method of the invention although the method could be carried out with other nematodes and in particular with other microscopic nematodes, preferably microscopic nematodes belonging to the genus *Caenorhabditis*. As used herein the term "microscopic" nematode encompasses nematodes of

approximately the same size as *C. elegans*, being of the order 1mm long in the adult stage. Microscopic nematodes of this approximate size can easily be grown in the wells of a multi-well plate of the type generally used in the art to perform mid- to high-throughput screening.

It is an essential feature of this aspect of the invention that the nematode has a non wild-type genetic background which confers greater sensitivity to RNA interference phenomena (abbreviated herein to RNAi) as compared to the equivalent wild type nematodes. As illustrated in the accompanying examples, introduction of double-stranded RNA (abbreviated herein to dsRNA) into a non wild-type strain according to the invention results in greater inhibition of expression of the target gene. Depending on the nature of the target gene, this greater level of inhibition may be detectable at the phenotypic level as a more pronounced phenotype.

The nematode having non wild-type genetic background may, advantageously, be a mutant strain. Mutations which have the effect of increasing susceptibility of the nematode to RNAi may, for example, affect the stability of dsRNA or the kinetics of dsRNA turnover within cells of the worm or the rate of uptake of dsRNA synthesised by a food organism. Suitable mutant strains include mutant strains exhibiting knock-out or loss-of-function mutations in one or more genes encoding proteins involved in RNA synthesis, RNA degradation or the regulation of these processes.

In one preferred embodiment, the nematode is a mutant strain, more preferably a mutant *C. elegans*, which exhibits reduced activity of one or more nucleases compared to wild-type. Suitable strains include mutant strains exhibiting knock-out or loss-of-function mutations in one or more genes encoding

nucleases, such as RNases. A particularly preferred example is the *nuc-1* strain. This mutant *C. elegans* strain is known *per se* in the art.

In a second preferred embodiment, the nematode is a mutant strain, more preferably a mutant *C. elegans*, which exhibits increased gut uptake compared to wild-type. Particularly preferred examples of such strains are the so-called *C. elegans* gun mutants described herein. In a still further embodiment, the nematode may be a transgenic worm comprising one or more transgenes which increase gut uptake relative to wild-type.

The term "increased gut uptake" as used herein is taken to mean increased uptake of foreign particles from the gut lumen and may encompass both increased gut permeability and increased gut molecular transport compared to wild-type *C. elegans*.

*C. elegans* feeds by taking in liquid containing its food (e.g. bacteria). It then spits out the liquid, crushes the food particles and internalises them into the gut lumen. This process is performed by the muscles of the pharynx. The process of taking up liquid and subsequently spitting it out is called pharyngeal pumping. Once the food particles have been internalised via pharyngeal pumping their contents must cross the gut itself in order to reach target sites in the worm. There are multiple factors which effect the uptake of compounds from the gut lumen to the surrounding tissues. These include the action of multi-drug resistance proteins, multi-drug resistance related proteins and the P450 cytochromes as well as other enzymes and mechanisms available for transport of molecules through the gut wall.

*C. elegans* mutants which exhibit increased uptake of foreign molecules through the gut may be obtained from the *C. elegans* mutant collection at the *C.*

*elegans* Genetic Center, University of Minnesota, St Paul, Minnesota, or may be generated by standard methods. Such methods are described by Anderson in Methods in Cell Biology, Vol 48, "*C. elegans*: Modern biological analysis of an organism" Pages 31 to 58. Several selection rounds of the PCR technique can be performed to select a mutant worm with a deletion in a desired gene. Alternatively, a population of worms could be subjected to random mutagenesis and worms exhibiting the desired characteristic of increased gut uptake selected using a phenotypic screen, such as the dye uptake method described herein.

As an alternative to mutation, transgenic worms may be generated with the appropriate characteristics. Methods of preparing transgenic worms are well known in the art and are particularly described by Craig Mello and Andrew Fire, Methods in Cell Biology, Vol 48, Ed. H.F. Epstein and D.C. Shakes, Academic Press, pages 452-480.

Worms exhibiting the desired characteristics of increased gut uptake can be identified using a test devised by the inventors based on uptake of a marker precursor molecule which is cleaved by the action of enzymes present in the gut lumen to generate a marker molecule which produces a detectable signal, such as fluorescence. A suitable marker precursor molecule is the fluorescent dye precursor BCECF-AM available from Molecular Probes (Europe BV), Netherlands. This dye only becomes fluorescent when cleaved by esterases and maintained at a pH above 6. The pH of the gut lumen is usually 5 or below. Thus, any BCECF-AM taken up through the pharynx into the gut lumen is not fluorescent until cleaved and the cleaved portion has entered the cells surrounding the lumen which are at a higher pH. Thus, this dye is able to quickly identify mutant or otherwise modified worms which have increased gut transport or permeability. There is a

gradual increase in fluorescence in the tissues surrounding the gut while the gut lumen remains dark. The fluorescence can be detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Specific examples of gun mutant strains isolated using this procedure which may be used in the method of the invention are strains bg77, bg84, bg85 and bg86, although it is to be understood that the invention is in no way limited to the use of these specific strains. The *C. elegans* gun mutant strain bg85 was deposited on 23 December 1999 at the BCCM/LMG culture collection, Laboratorium Voor Microbiologie, Universiteit Gent, K. L. Ledeganckstraat 35, B-9000, Gent, Belgium under accession number LMBP 5334CB. The phrase "the bg85 mutation" as used herein refers to the specific mutation(s) present in the bg85 strain which is/are responsible for conferring the gun phenotype.

It is also within the scope of the invention to use a non wild-type nematode strain, preferable a *C. elegans* strain, having multiple mutations which affect sensitivity to RNAi. A preferred type of multiple mutant is one having at least one mutation which results in reduced nuclease activity compared to wild type and at least one mutation which results in increased gut uptake compared to wild type. An example of such a mutant is a *C. elegans* strain having the *nuc-1* mutation and at least one further gun mutation. As exemplified herein, double mutants having the *nuc-1* mutation and a gun mutation exhibit enhanced sensitivity to RNAi as compared to either *nuc-1* or gun single mutants.

For the avoidance of doubt, where particular characteristics or properties of nematode worms are described herein by relative terms such as "enhanced"

or "increased" or "decreased" this should be taken to mean enhanced, increased or decreased relative to wild-type nematodes. In the case of *C. elegans*, wild-type is defined as the N2 Bristol strain which is well known to workers in the *C. elegans* field and has been extremely well characterised (see Methods in Cell Biology, Volume 48, Caenorhabditis elegans: Modern biological analysis of an organism, ed. by Henry F. Epstein and Diane C. Shakes, 1995 Academic Press; The nematode Caenorhabditis elegans, ed. by William Wood and the community of *C. elegans* researchers., 1988, Cold Spring Harbor Laboratory Press; *C. elegans* II, ed. by Donald L. Riddle, Thomas Blumenthal, Barbara J. Meyer and James R. Priess, 1997, Cold Spring Harbor Laboratory Press). The N2 strain can be obtained from the *C. elegans* Genetic Center, University of Minnesota, St Paul, Minnesota, USA.

The food organism for use in the above aspect of the invention is preferably a bacterium such as, for example, a strain of *E.coli*. It will, however, be appreciated that any other type of food organism on which nematodes feed and which is capable of producing dsRNA could be used. The food organism may be genetically modified to express a double-stranded RNA of the appropriate sequence, as will be understood with reference to the examples included herein. One convenient way in which this may be achieved in a bacterial food organism is by transforming the bacterium with a vector comprising a promoter or promoters positioned to drive transcription of a DNA sequence to RNA capable of forming a double-stranded structure. Examples of such vectors will be further described below.

The actual step of feeding the food organism to the nematode may be carried out according to procedures known in the art, see WO 00/01846.

Typically the feeding of the food organisms to the nematodes is performed on standard agar plates commonly used for culturing *C. elegans* in the laboratory. However, the step of feeding the food organism to the nematodes may also be carried out in liquid culture, for example in the wells of 96-well microtitre assay plates.

The inventors have further observed that variations in the food organism can result in enhanced *in vivo* RNAi when the food organism is ingested by a nematode worm.

Accordingly, in a further aspect the invention provides a method of inhibiting expression of a target gene in a nematode worm comprising feeding to said nematode worm a food organism capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a portion of said target gene following ingestion of the food organism by the nematode, wherein the food organism carries a modification selected to provide increased expression or persistence of the doubled-stranded RNA compared to a food organism which does not carry the modification.

The modification present in the food organism can be any modification which results in increased expression of the dsRNA or in increased persistence of the dsRNA. Suitable modifications might include mutations within the bacterial chromosome which affect RNA stability and/or degradation or mutations which have a direct effect on the rate of transcription. In a preferred embodiment, the food organism is an RNAse III minus *E. coli* strain, or any other RNAse negative strain.

According to a still further aspect of the invention there is provided a method of inhibiting expression of a target gene in a nematode worm comprising introduction of a DNA capable of producing a double-stranded RNA structure having a nucleotide

sequence substantially identical to a portion of said target gene in said nematode, wherein the nematode is one which exhibits increased gut uptake compared to wild type.

In addition to exhibiting increased sensitivity to RNAi following feeding with food organisms capable of expressing a dsRNA, nematodes which exhibit increase gut uptake as described herein also show increased uptake of DNA molecules capable of producing double-stranded RNA structures following ingestion into a nematode.

In a preferred embodiment, the DNA is in the form of a vector comprising a promoter or promoters orientated to relative to a sequence of DNA such that they are capable of driving transcription of the said DNA to make RNA capable of forming a double-stranded structure upon binding of an appropriate RNA polymerase to the promoter or promoters.

Several different arrangements of promoters may be used in such a vector. In a first arrangement a DNA fragment corresponding to a region of the target gene is flanked by two opposable polymerase-specific promoters which are preferably identical. Transcription from the opposable promoters produces two complementary RNA strands which can anneal to form an RNA duplex. The plasmid pGN1 described herein is an example of a vector comprising two opposable T7 promoters flanking a multiple cloning site for insertion of a DNA fragment of the appropriate sequence, corresponding to a region of a target gene. pGN8 is an example of a vector derived from pGN1 containing a fragment of the *C. elegans unc-22* gene. In an alternative arrangement, DNA fragments corresponding to a region of the target gene may be placed both in the sense and the antisense orientation downstream of a single promoter. In this case, the sense/antisense fragments are co-transcribed to

generate a single RNA strand which is self-complementary and can therefore form an RNA duplex.

In both of the above arrangements, the polymerase-specific T3, T7 and SP6 promoters, all of which are well known in the art, are useful for driving transcription of the RNA. Expression from these promoters is dependent on expression of the cognate polymerase. Advantageously, the nematode itself may be adapted to express the appropriate polymerase. Expression of the polymerase may be general and constitutive, but could also be regulated under a tissue-specific promoter, an inducible promoter, a temporally regulated promoter or a promoter having a combination of such characteristics. Transgenic *C. elegans* strains harboring a transgene encoding the desired polymerase under the control of an appropriately-regulated promoter can be constructed according to methods known *per se* in the art and described, for example, by Craig Mello and Andrew Fire in Methods in Cell Biology, Vol 48, Ed. H. F. Epstein and D. C. Shakes, Academic Press, pp 452-480.

The advantage of adapting the nematode to express the required polymerase is that it is possible to control inhibition of expression of the target gene in a tissue-specific and/or temporally specific manner by placing expression of the polymerase under the control of an appropriately regulated promoter.

Introduction of DNA into nematodes in accordance with the method of the invention can be achieved using a variety of techniques, for example by direct injection into a body cavity or by soaking the worms in a solution containing the DNA. If the DNA is in the form of a vector as described herein, e.g. a plasmid harboring a cloned DNA fragment between two flanking T7 promoters, then dsRNA corresponding to this DNA fragment will be formed in the nematode resulting in down regulation of the corresponding gene. The

introduced DNA can form an extrachromosomal array, which array might result in a more catalytic knock-out or reduction of function phenotype. The DNA might also become integrated into the genome of the nematode, resulting in the same catalytic knock out or reduction of function phenotype, but which is stably transmittable.

In each aspect of the invention, the double-stranded RNA structure may be formed by two separate complementary RNA strands or a single self-complementary strand, as described above. Inhibition of target gene expression is sequence-specific in that only nucleotide sequences corresponding to the duplex region of the dsRNA structure are targeted for inhibition.

It is preferred to use dsRNA comprising a nucleotide sequence identical to a portion of the target gene, although RNA sequences with minor variations such as insertions, deletions and single base substitutions may also be used and are effective for inhibition. It will be readily apparent that 100% sequence identity between the dsRNA and a portion of the target gene is not absolutely required for inhibition and the phrase "substantially identical" as used herein is to be interpreted accordingly. Generally sequences which are substantially identical will share at least 90%, preferably at least 95% and more preferably at least 98% nucleic acid sequence identity. Sequence identity may be conveniently calculated based on an optimal alignment, for example using the BLAST program accessible at WWW.ncbi.nlm.nih.gov.

The invention will be further understood with reference to the following non-limiting Examples, together with the accompanying Figures in which:

Figure 1 is a plasmid map of the vector pGN1

containing opposable T7 promoters flanking a multiple cloning site and an ampicillin resistance marker.

Figure 2 is a plasmid map of the vector pGN8 (a genomic fragment of the *C. elegans unc-22* gene cloned in pGN1).

Figure 3 is a plasmid map of the vector pGN29 containing two T7 promoters and two T7 terminators flanking *Bst*XI sites. This vector permits cloning of DNA fragments linked to *Bst*XI adaptors.

Figure 4 is a plasmid map of the vector pGN39 containing two T7 promoters and two T7 terminators flanking attR recombination sites (based on the Gateway™ cloning system of Life Technologies, Inc).

Figure 5 is a plasmid map of the vector pGX22 (a fragment of the *C. elegans* gene C04H5.6 cloned in pGN29).

Figure 6 is a plasmid map of the vector pGX52 (a fragment of the *C. elegans* gene K11D9.2b cloned in pGN29).

Figure 7 is a plasmid map of the vector pGX104 (a fragment of the *C. elegans* gene Y57G11C.15 cloned in pGN29).

Figure 8 is a plasmid map of the vector pGZ8 (a fragment of the *C. elegans* gene T25G3.2 cloned in pGN39).

Figure 9 shows the results of an RNAi experiment in which wild-type (N2) or *nuc-1* strain *C. elegans* in liquid culture were fed with *E. coli* containing the

plasmid pGX22.

Figure 10 shows the results of an RNAi experiment in which wild-type (N2) or *nuc-1* strain *C. elegans* in liquid culture were fed with *E. coli* containing the plasmid pGX52.

Figure 11 shows the results of an RNAi experiment in which wild-type (N2) or *nuc-1* strain *C. elegans* in liquid culture were fed with *E. coli* containing the plasmid pGXGZ8.

Figure 12 shows the results of an RNAi experiment in which wild-type (N2) or *nuc-1* strain *C. elegans* in liquid culture were fed with *E. coli* containing the plasmid pGX104

**Example 1**

Influence of genetic background on the efficiency of RNAi in *C. elegans*.

**Introduction**

Various different *C. elegans* strains were fed with different bacteria, to test the possibility of RNAi by feeding *C. elegans* with bacteria that produce dsRNA. The possibility of DNA delivery and dsRNA delivery has previously been envisaged by using different bacterial strains. In this experiment the importance of the *C. elegans* strain as receptor of the dsRNA is also shown.

For this experiment the following *E. coli* strains were used:

1. MC1061: F-*araD139 Δ(ara-leu)7696 galE15 galK16 Δ(lac)X74 rpsl (Str$^r$) hsdR2 ($r_k^-$ $m_k^+$) mcrA mcrB1*
   - regular host for various plasmids,
   - Wertman et al., (1986) Gene 49:253-262,
   - Raleigh et al., (1989) in Current Protocols in Molecular Biology eds. Ausubel et al, Publishing associates and Wiley Interscience; New York. Unit 1.4.

2. B21(DE3): F- *ompT(lon) hsdS$_B$* ($r_B^-$,$m_B^-$; an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene.
   - regular host for IPTG inducible T7 polymerase expression,
   - Studier et al. (1990) Meth. Enzymol. 185:60-89

3. HT115 (DE3): F- *mcrA mcrb* IN(rrnD-rrnE) 1 λ- *rnc14::tr10* (DE3 lysogen: lacUV5 promoter-T7polymerase)
   - host for IPTG inducible T7 polymerase

expression,
- RNaseIII-,
- Fire A, Carnegie Institution, Baltimore, MD, Pers. Comm.

For this experiment the following *C. elegans* strains were used:

1. C. elegans N2: regular WT laboratory strain

2. *C. elegans nuc-1*(e1393): *C. elegans* strain with a reduced endonuclease activity (>95%); condensed chromatin persists after programmed cell death; ingested (bacterial) DNA in the intestinal lumen is not degraded. Several alleles are described: e1392 (strong allele: has been used for the experiments described below); n887 (resembles e1392) and n334 (weaker allele)
   - Stanfield et al. (1998) East Coast Worm meeting abstract 171,
   - Anonymous, Worm Breeder's Gazette 1(1):17b Hevelone et al. (1988) Biochem. Genet. 26:447-461
   - Ellis et al., Worm breeder's Gazette 7(2):44
   - Babu, Worm Breeder's gazette 1(2):10
   - Driscoll, (1996) Brain Pathol. 6:411-425
   - Ellis et al., (1991) Genetics 129:79-94

For this experiment the following plasmids were used:

pGN1: A vector encoding for ampicillin resistance, harbouring a multiple cloning site between two convergent T7 promoters.

pGN8: pGN1 containing a genomic fragment of *unc-22*. Decreased unc-22 expression via RNAi results in a "twitching" phenotype in *C. elegans*.

**Experimental conditions**

12-well micro-titer plates were filled with approximately 2 ml of NGM agar per well (1 litre of NGM agar: 15g Agar, 1g peptone, 3g NaCl, 1ml cholesterol solution (5 mg/ml in EtOH), with sterile addition after autoclaving of 9.5 ml 0.1M $CaCl_2$, 9.5 ml 0.1 ml $MgSO_4$, 25 ml 1M $KH_2PO_4/K_2HPO_4$ buffer pH 6 and 5 ml nystatin solution (dissolved 10 mg/ml in 1:1 EtOH:$CH_3COONH_4$ 7.5 M).

The dried plates were spotted with approximately 50 µl of an overnight culture of bacteria. When IPTG induction was required, 50 µl of a 10 mM stock solution of IPTG was dropped on top of the bacteria lawn, and incubated at 37°C for approximately 4 hours. Individual nematodes at the L4 growth stage were then placed in single wells. In each well 4 nematodes, and the plates were further incubated at 20°C for 6 days to allow offspring to be formed. The F1 offspring of the seeded nematodes were tested for the twitching phenotype.

**Results**

Table 1: Percentage of the offspring that show the twitching phenotype

| **MC1061** | **N2** | ***nuc-1*** |
|---|---|---|
| pGN1 | 0% | 0% |
| pGN1 + IPTG | 0% | 0% |
| pGN8 | 0% | 0% |
| pGN8 + IPTG | 0% | 0% |
| **BL21(DE3)** | | |
| pGN1 | 0% | 0% |
| pGN1 + IPTG | 0% | 0% |
| pGN8 | 20% (+) | >90% (++) |
| pGN8 + IPTG | 20% (+) | >90% (++±) |
| **HT115(DE3)** | | |
| pGN1 | 0% | 0% |
| pGN1 + IPTG | 0% | 0% |
| pGN8 | 50% (+±) | >90% (++) |
| pGN8 + IPTG | 80% (++) | >90% (+++) |

%: percentage twitchers
+: weak twitching
++: twitching
+++: strong twitching

**Conclusions**

The experiment with *E. coli* MC1061 shows that no twitching could be observed in this experiment. Neither the N2 nematodes nor the *nuc-1* nematodes showed any twitchers. This is to be expected as *E. coli* MC1061 does not produce any T7 RNA polymerase, and hence the unc-22 fragment cloned in pGN8 is not

expressed as dsRNA.

The experiment with *E. coli* strain BL21(DE3) and nematode strain N2 shows expected results. BL21(DE3) harbouring plasmid pGN1 does not result in any twitching as the pGN1 vector is an empty vector. BL21 (DE3) harbouring PGN8 results in the expression of unc-22 dsRNA. When this dsRNA is fed to the N2 nematode (indirectly by feeding with the bacteria that produce the dsRNA), this results in a twitching phenotype, indicating that the dsRNA is able to pass the gut barrier and is able to perform its interfering activity.

Surprisingly the RNAi effect of the unc-22 dsRNA was even more pronounced in *C. elegans* strain *nuc-1* than in the wild type N2 strain. Although one may expect that the *nuc-1* mutation results in the non-degradation or at least in a slower degradation of DNA, as the NUC-1 protein is known to be involved in DNAse activity, we clearly observe an enhancement of the RNAi induced phenotype in *C. elegans* with a *nuc-1* background. The *nuc-1* mutation has not been cloned yet, but it has been described that the gene is involved in nuclease activity, and more particularly DNAse activity. If the NUC-1 protein is a nuclease, it may also have activity on nuclease activity on dsRNA, which would explain the enhanced RNAi phenotype. The *nuc-1* gene product may be a nuclease, or a regulator of nuclease activity. As the mode of action of RNAi is still not understood, it is also possible that the NUC-1 protein is interfering in the mode of action of RNAi. This would explain why a *nuc-1* mutant is more sensitive to RNAi.

The experiment with the *E.coli* strain HT115 (DE3)

confirms the experiments with the BL21(DE3) strain. The RNA interference observed with the unc-22 dsRNA is even higher. In comparison with strain BL21(DE3) this could be expected, as HT115(DE3) is a RNAse III minus strain, and hence is expected to produce larger amounts of dsRNA, resulting in more prominent RNAi. This indicates further that the RNAi observed in this experiment is the result of the dsRNA produced by the bacteria fed to the *C. elegans*. Feeding *C. elegans nuc-1* with HT115(DE3) harbouring pGN8 also results in higher RNA interference phenotype than feeding the same bacteria to *C. elegans* wild-type strain N2. Once again this indicates that improved RNAi can realised using a nuclease negative *C. elegans* and more particularly with a with the *C. elegans nuc-1* (e1392) strain.

**Summary**

RNA interference can be achieved in *C. elegans* by feeding the worms with bacteria that produce dsRNA. The efficiency of this RNA interference is dependent both on the *E. coli* strain and on the genetic background of the *C. elegans* strain. The higher the level of dsRNA production in the *E. coli*, the more RNAi is observed. This can be realised by using efficient RNA expression systems such as T7 RNA polymerase and RNAase negative strains, such as RNaseIII minus stains. In this example the level of dsRNA production varied: HT115(DE3)>BL21(DE3)>MC1061.

RNA interference is high in *C. elegans* strains that are nuclease negative, or that are influenced in their nuclease activity. This can be realised by using a mutant strain such as *C. elegans nuc-1*.
In this example the sensitivity to RNAi varied:
C. elegans nuc-1 >> C. elegans N2

**Example 2**

Improved RNAi by feeding dsRNA producing bacteria in selected *C. elegans* strains-Comparison of the *nuc-1* strain with several mutants which show improved gut uptake (designated herein 'gun' mutants). Strains bg77, bg78, bg83, bg84, bg85, bg86, bg87, bg88 and bg89 are typical gun mutant *C. elegans* strains isolated using selection for increased gut uptake (gun phenotype) with the marker dye BCECF-AM.

**Experimental conditions:**

- 12-well micro-titer plates were filled with approximately 2ml of NGM agar (containing 1ml/l of ampicillin (100µg/ml) and 5 ml of 100mM stock IPTG) per well
- the dried plates were spotted with 25µl of an overnight culture of bacteria (BL21DE3/HT115DE3) containing the plasmids pGN1 (T7prom-T7prom) or pGN8 (T7prom-unc-22-T7prom)
- individual nematodes at the L4 growth stage were then placed in single wells, one nematode per well
- the plates were incubated at 20°C for 6 days to allow offspring to be formed
- the adult F1 offspring of the seeded nematodes were tested for the twitching phenotype

**Results:**

Table 2:

| 20°C/6d | pGN1 HT115DE3 | pGN8 BL2DE3 | pGN8 HT115DE3 | |
|---|---|---|---|---|
| N2 | 0 | 1 | 1 | |
| *nuc-1* | 0 | 1-2 | 3 | |
| bg77 | 0 | 1-2 | 3 | |
| bg78 | 0 | 1 | 1-2 | |
| bg83 | 0 | 1 | 1 | |
| bg84 | 0 | 1-2 | 3 | |
| bg85 | 0 | 1 | 2-3 | |
| bg86 | 0 | 1 | 2 | |
| bg87 | 0 | 1 | 1 | |
| bg88 | 0 | 1 | 1 | |
| bg89 | 0 | 1 | 1 | |
| | | | | |

figure legend:

0 = no twitching

1 = no to weak phenotype

2 = clear phenotype

3 = strong phenotype

**Conclusions**

- bacterial strain HT115(DE3) shows a better RNAi sensitivity than bacterial strain BL21(DE3)
- the *nuc-1 C. elegans* strain is a better strain than the Wild-type N2 strain for RNAi sensitivity
- various gun mutants (improved gut uptake mutants) and more particularly the gun mutant strains bg77, bg84, bg85, bg86 show improved sensitivity to RNAi compared to Wild-type.

**A double mutant *C. elegans* strain (nuc-1/gun) shows even greater sensitivity to RNAi compared to wild-type:**

Double mutants were constructed to test the prediction that gun/nuc mutants would even show more enhanced RNAi sensitivity. As an example, the crossing strategy with gun strain bg85 is shown, similar crosses can be conducted with other gun strains, such as bg77, bg84 and bg86.

P0 cross: gun(bg85) x WT males

F1 cross: nuc-1 x gun(bg85)/+ males

F2 cross: nuc-1 x gun(bg85)/+; nuc-1/0 males (50%)
nuc-1 x +/+; nuc-1/0 males (50%)

F3 single: gun(bg85)/+; nuc-1 hermaphrodites (25%)
+/+; nuc-1 hermaphrodites (75%)

F4 single: gun(bg85); nuc-1 (1/4 of every 4th plate high staining with BCECF)

F5 retest: gun(bg85); nuc-1 (100% progeny of F4 singled high staining with BCECF)

To select for the gun phenotype, the fluorescence precursor BCECF-AM is used (obtainable from Molecular probes). The precursor BCECF-AM is cleaved by esterases present in the gut of the worm to generate the dye BCECF which is fluorescent at pH values above 6. This allows selection for worms that have a gun phenotype. BCECF-AM is taken up through the pharynx into the gut lumen and is not fluorescent until it has been cleaved, and the BCECF portion has entered the

cells surrounding the lumen. Wild-type worms will show slower or no increase in BCECF fluorescence.

**Example 3**

Improved RNAi feeding in liquid culture using *nuc-1*(e1393) *C. elegans*.

**Introduction**

N2 and nuc-1 *C.elegans* strains were fed with bacteria producing dsRNAs that give lethal phenotypes via RNAi. For this example RNAi was performed in liquid culture instead of on agar plates. We show here for a number of genes that the RNAi effect is more penetrant using the nuc-1 strain than the N2 strain, and that RNAi can be performed in liquid.

For this experiment the following *E.coli* strains were used:

1. HT115 (DE3): F- *mcrA mcrb IN*(rrnD-rrnE) 1 λ- *rnc14::tr10* (DE3 lysogen: lacUV5 promoter -T7 polymerase)
   - host for IPTG inducible T7 polymerase expression
   - RNaseIII$^-$
   - Fire A, Carnegie Institution, Baltimore, MD, Pers. Comm.

For this experiment, following *C. elegans* strains were used:

1. *C. elegans* N2: regular WT laboratory strain

2. *C. elegans nuc-1*(e1393): *C. elegans* strain with a reduced endonuclease activity (>95%); condensed chromatin persists after programmed cell death;

ingested (bacterial) DNA in the intestinal lumen is not degraded. Several alleles are described: e1392 (strong allele: has been used for the experiments described below); n887 (resembles e1392) and n334 (weaker allele)

- Stanfield et al. (1998) East Coast Worm meeting abstract 171
- Anonymous, Worm Breeder's Gazette 1(1):17b
- Hevelone et al. (1988) Biochem. Genet. 26:447-461
- Ellis et al., Worm breeder's Gazette 7(2):44
- Babu, Worm Breeder's gazette 1(2):10
- Driscoll, (1996) Brain Pathol. 6:411-425
- Ellis et al., (1991) Genetics 129:79-94

For this experiment, the following plasmids that all give lethal phenotypes in *C. elegans* via RNAi were used:

pGX22: a vector encoding ampicillin resistance, containing a genomic fragment of cosmid C04H5.6 corresponding to a member of the RNA helicase family.

pGX52: a vector encoding ampicillin resistance, containing a genomic fragment of cosmid K11D9.2b corresponding to sarco/endoplasmic $Ca2^{+}$ ATPase also known as SERCA.

pGZ18: a vector encoding ampicillin resistance, containing a genomic fragment of cosmid T25G3.2 corresponding to a chitin like synthase gene.

pGX104: a vector encoding ampicillin resistance, containing a genomic fragment of cosmid Y57G11C.15 corresponding to sec-61, a transport protein.

**Experimental conditions**

- 1 ml overnight cultures of HT115 (DE3) bacteria containing the plasmids pGX22, pGX52, pGZ18 or pGX104 respectively were pelleted and resuspended in S-complete medium, containing 1ml/l of ampicillin (100 µg/ml) and 1ml/l of 1000mM IPTG.

- 10 µl of this bacterial solution was transferred to a 96-well microtiter plate already filled with 100 µl S-complete containing 1ml/l of ampicillin (100 µg/ml) and 1ml/l of 1000mM IPTG.

- 3 nematodes at the L1 growth stage of N2 and nuc-1 strain were then placed in single wells, 3 L1's per well. Per experimental set up, 16 wells were used (n=16).

- the plates were incubated at 25°C for 5 days to allow offspring to be formed.

- the plates were visually checked and the following phenotypes could be scored per individual well:

**no effect:** L1's developed to adults and gave normal offspring.

**no F1 offspring:** L1's developed to adults and gave no offspring.

**acute lethal:** original L1 did not mature and died.

**Results**

The results of this experiment are illustrated graphically in Figures 9 to 12. Data are expressed as

a percentage of the total (n=16) on the y-axis for both N2 and *nuc-1* strains.

**Conclusions**

The following genes were tested in this liquid RNAi assay:

- C04H5.6: an RNA helicase. RNAi of this gene interferes with the generation of offspring.
- SERCA: a sarco/endoplasmic $Ca^{2+}$ ATPase. A strong RNAi phenotype causes an acute lethal phenotype. A less penetrant RNAi effect results in loss of offspring.
- T25G3.2: a chitin like synthase gene. RNAi of this gene causes dead eggs.
- sec-61: a transport protein. A strong RNAi phenotype causes an acute lethal phenotype. A less penetrant RNAi effect results in loss of offspring.
- RNAi can be performed under liquid conditions.

As in the previous examples this set of experiments shows that the nuc-1 *C. elegans* strain is more sensitive to RNAi than the wild-type N2 strain. This is most clear for less penetrant phenotypes such as SERCA and chitin synthase. For strong RNAi phenotypes like the helicase and Sec-61 the difference between the N2 wild-type strain and the nuc-1 stain is less pronounced.

**Example 4**

**Cloning of pGX22, pGX52, pGZ18 and pGX104 for RNAi**

A set of primers for each gene was designed on the basis of sequence data available in the publicly accessible *C. elegans* sequence database (Acedb).

The cosmid names relate to:

1. **C04H5.6**=member of RNA helicase
2. **K11D9.2b**=SERCA
3. **Y57G11C.15**=transport protein sec-61
4. **T25G3.2**=chitin synthase like

The following primer sequences were designed:

1. **C04H5.6F** 5'-TGCTCAGAGAGTTTCTCAACGAACC-3'
   **C04H5.6R** 5'-CAATGTTAGTTGCTAGGACCACCTG-3'

2. **K11D9.2bF** 5'-CAGCCGATCTCCGTCTTGTG-3'
   **K11D9.2bR** 5'-CCGAGGGCAAGACAACGAAG-3'

3. **Y57G11C.15F** 5'-ACCGTGGTACTCTTATGGAGCTCG-3'
   **Y57G11C.15R** 5'-TGCAGTGGATTGGGTCTTCG-3'

4. **T25G3.2F**
   5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCCAAGTACATGTCGATTGCG-3'

   **T25G3.2R**
   5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTGGAGAAGCATTCCGAGAGTTTG-3'

PCR was performed on genomic DNA of N2 strain *C. elegans* to give PCR products of the following sizes:

1326bp for C04H5.6
1213bp for K11D9.2b

1024bp for Y57G11C.15
1115bp for T25G3.2

The PCR fragments of C04H5.6, K11D9.2b and Y57G11C.15 were linked to *Bst*XI adaptors (Invitrogen) and then cloned into the pGN29 vector cut with *Bst*XI. pGN29 contains two T7 promoters and two T7 terminators flanking a cloning site which is adapted for facilitated cloning of PCR fragments, comprising a stuffer DNA flanked by two *Bst*XI sites (see schematic Figure 3). The resulting plasmids were designated pGX22 (C04H5.6), pGX52 (K11D9.2b) and pGX104 (Y57G11C.15).

The PCR fragment of T25G3.2 was cloned into pGN39 via recombination sites based on the GATEWAY™ cloning system (Life Technologies, Inc). pGN39 contains two T7 promoters and two T7 terminators flanking a cloning site which facilitates "High Throughput" cloning based on homologous recombination rather than restriction enzyme digestion and ligation. As shown schematically in Figure 4, the cloning site comprises *att*R1 and *att*R2 recombination sites from bacteriophage lambda flanking a gene which is lethal to *E. coli*, in this case the *ccd*B gene. This cloning site is derived from the Gateway™ cloning system commercially available from Life Technologies, Inc. The Gateway™ cloning system has been extensively described by Hartley *et al.* in WO 96/40724 (PCT/US96/10082).

**Example 5**

Selecting *C. elegans* mutations for increased gut uptake (gun) using marker dye BCECF-AM and *unc-31* as background.

The screen was performed in *unc-31*(*e928*) mutant background, to ensure high amounts of dye in the gut lumen, since *unc-31* mutations show constitutive pharyngeal pumping. The dye (BCECF-AM: 2',7' bis (2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethylester), obtained from Molecular Probes, is cleaved by intracellular esterases. Fluorescence accumulates in the gut cells upon passage through the apical gut membrane.

Mutagenesis

Day 1: *unc-31* L4 staged worms were mutagenised with EMS (final concentration 50 mM) for 4 hours

Day 2: P0 was divided over several large agar plates

Day 6: F1's were collected and dropped on large plates. The number of eggs the F1's layed were checked every hour and de F1's were removed when 10-20 eggs per F1 were counted

Day 10: F2 adults were collected and screened with BCECF-AM. Mutations with increased staining of the gut cells after 15-30 minutes exposure to the dye were selected and singled on small agar plates.

About 50 initial positives gave progeny which was retested with BCECF-AM (2x) and leucine CMB (1x)
9 of the 50 strains were kept (2 strains : 3 times positive, 7 other strains : twice positive)

Table 3: Isolation of mutations for increased staining with BCECF-AM

| Total P0 | Total F1 | Total F2 | screened chromosomes | number of strains isolated |
|---|---|---|---|---|
| (counted) | (estimated) | (calculated) | (estimated) | (counted) |
| 2251 | 55618 | 222472 | 100000 | 9 |

Outcrossing, backcrossing and double construction

1. backcrossing *unc-31; gun* --> *unc-31; gun*
- *unc-31; gun* x WT males
- singled 2x5 WT hermaphrodites F1s (*unc-31/+;gun/+*)
- singled 50 WT hermaphrodites F2s (1/4 homozygous)
- select strains segregating 1/4 unc
- stain unc strains with BCECF-AM
- from positive strains pick unc homozygous
- retest 100 % unc strains with BCECF-AM
- kept 1 strain (backcrossed)

2. *unc-31* background was crossed out-->+; *gun*
- *unc-31; gun* x WT males
- singled 2x5 WT hermaphrodites F1s (*unc-31/+;gun/+*)
- singled 50 WT hermaphrodites F2s (1/4 homozygous)
- select strains which did not segregate unc F3s anymore
- stain non unc strains with BCECF-AM
- 7 positive strains were retested with BCECF-AM and finally 1 was selected and kept (outcrossed)

3. +; *gun* (1x outcrossed) were 2 times backcrossed-->+; *gun* (3x backcrossed)
- *gun* x WT males
- WT hermaphrodites x F1 males (*gun/+*)
- singled 10 WT hermaphrodites F2s (½ heterozygous)
- singled 50 WT hermaphrodites F3s (1/8 homozygous)

- stain strains with BCECF-AM- retested positives with BCECF-AM and finally 1 was selected and kept

4. *gun* (3x backcrossed) were crossed with *nuc-1*(X) mutant--> *gun; nuc-1*
- *gun* x WT males
- *nuc-1* x *gun/+* males
- *nuc-1* x *gun/+; nuc-1/0* or *+/+; nuc-1/0* males
- singled 10 WT hermaphrodite progeny (*nuc-1* homozygous, ½ heterozygous *gun*)
- singled 40 WT hermaphrodite progeny (1/8 homozygous *gun*)
- stain strains with BCECF-AM
- retested positives with BCECF-AM and finally 1 was selected and kept

Table 6: Strains derived from gun mutations

| gun | *unc-31; gun* | | *unc-31; gun* | | *+; gun* | | | *gun; nuc-1* |
|---|---|---|---|---|---|---|---|---|
| | original isolate | | backcrossed (1x) | | outcrossed (1x) | | 3x b.c. | from 3x b.c. |
| allele number | isolation number | strain number | isolation number | strain number | isolation number | strain number | strain number | strain number |
| bg77 | 31.4 | UG 510 | 31.4.46.1 | UG 556 | 31.4.34 | UG 563 | UG 674 | UG 777 |
| bg78 | 37.5 | UG 511 | 37.5.46.4 | UG 557 | 37.5.15 | UG 564 | UG 675 | – |
| bg83 | 10.2 | UG 543 | 10.2.11 | UG 600 | 10.2.21 | UG 586 | UG 676 | – |
| bg84 | 7.2 | UG 544 | 7.2.10 | UG 601 | 7.2.15 | UG 589 | UG 677 | UG 774 |
| bg85 | 11.5 | UG 545 | 11.5.29.2 | UG 602 | 2x b.c. | UG 717 | | UG 775 |
| bg86 | 42.1 | UG 546 | 42.1.4.5 | UG 603 | 42.1.18 | UG 587 | UG 678 | UG 776 |
| bg87 | 7.1 | UG 547 | 7.1.8.3 | UG 604 | 7.1.22 | UG 585 | UG 679 | – |
| bg88 | 5.3 | UG 548 | 5.3.9 | UG 605 | 5.3.18 | UG 584 | UG 680 | – |
| bg89 | 23.4 | UG 549 | 23.4.13.5 | UG 606 | 23.4.3 | UG 588 | UG 671 | – |

SEQUENCE LISTING:

SEQ ID NO: 1 complete sequence of pGN1

SEQ ID NO: 2 complete sequence of pGN8

SEQ ID NO: 3 complete sequence of pGN29

SEQ ID NO: 4 complete sequence of pGN39

SEQ ID NO: 5 complete sequence of pGX22

SEQ ID NO: 6 complete sequence of pGX52

SEQ ID NO: 7 complete sequence of pGX104

SEQ ID NO: 8 complete sequence of pGZ8

SEQ ID NO: 9 primer C04H5.6F

SEQ ID NO: 10 primer C04H5.6R

SEQ ID NO: 11 primer K11D9.2bF

SEQ ID NO: 12 primer K11D9.2bR

SEQ ID NO: 13 primer Y57G11C.15F

SEQ ID NO: 14 primer Y57G11C.15R

SEQ ID NO: 15 primer T25G3.2F

SEQ ID NO: 16 primer T25G3.2R

**Claims:**

1. A method of inhibiting expression of a target gene in a nematode worm comprising feeding to said nematode worm a food organism which is capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a portion of said target gene following ingestion of the food organism by the nematode, wherein the nematode has a non wild-type genetic background selected to provide increased sensitivity to RNA interference as compared to wild type.

2. A method as claimed in claim 1 wherein the nematode is a microscopic nematode.

3. A method as claimed in claim 2 wherein the nematode is from the genus *Caenorhabditis*.

4. A method as claimed in claim 3 wherein the nematode is *C. elegans*.

5. A method as claimed in any one of claims 1 to 4 wherein the nematode has a mutant genetic background.

6. A method as claimed in claim 5 wherein the nematode is a mutant strain which exhibits reduced activity of one or more nucleases compared to wild type.

7. A method as claimed in claim 6 wherein the nematode is *C. elegans* strain *nuc-1*.

8. A method as claimed in claim 5 wherein the nematode is a mutant strain which exhibits increased

gut uptake compared to wild type.

9. A method as claimed in claim 8 wherein the nematode is mutant *C. elegans* strain bg85.

10. A method as claimed in claim 5 wherein the nematode is a mutant strain having at least one mutation which results in reduced nuclease activity compared to wild type and at least one mutation which results in increased gut uptake compared to wild type.

11. A method as claimed in claim 10 wherein the nematode is a mutant *C. elegans* strain having the *nuc-1* mutation and the bg85 mutation.

12. A method as claimed in any one of the preceding claims wherein the food organism has been engineered to express a double-stranded RNA.

13. A method as claimed in any one of the preceding claims wherein the food organism is a bacterium.

14. A method as claimed in claim 13 wherein the food organism is *E. coli*.

15. A method as claimed in any one of the preceding claims wherein the food organism has been genetically modified to express a double-stranded RNA having a nucleotide sequence substantially identical to a portion of said target gene.

16. A method as claimed in claim 15 wherein the food organism contains a DNA vector, the vector comprising a promoter or promoters orientated relative to a DNA sequence such that they are capable of

initiating transcription of said DNA sequence to RNA capable of forming a double-stranded structure upon binding of an appropriate RNA polymerase to said promoter or promoters.

17. A method as claimed in claim 25 wherein the vector comprises two promoters flanking the DNA sequence.

18. A method as claimed in claim 26 wherein the two promoters are identical.

19. A method as claimed in claim 25 wherein the vector comprises a single promoter and further comprises said DNA sequence in a sense and an antisense orientation relative to said promoter.

20. A method as claimed in any one of claims 16 to 20 wherein the nematode or the food organism is adapted to express an RNA polymerase capable of initiating transcription from said promoter or promoters.

21. A method as claimed in any one of claims 16 to 20 wherein the RNA polymerase is T7, T3 or SP6 polymerase.

22. A method as claimed in any one of claims 1 to 21 wherein the step of feeding said food organism to said nematode worm is carried out in liquid culture.

23. A method of inhibiting expression of a target gene in a nematode worm comprising feeding to said nematode worm a food organism capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a

portion of said target gene following ingestion of the food organism by the nematode, wherein the food organism carries a modification selected to provide increased expression or persistence of the double-stranded RNA compared to a food organism which does not carry the modification.

24. A method as claimed in claim 23 wherein the food organism is a bacterium.

25. A method as claimed in claim 24 wherein the bacterium is an *E. coli* strain.

26. A method as claimed in claim 25 wherein the *E. coli* strain is an RNAse III minus strain or any other RNAse negative strain.

27. A method as claimed in any one of claims 23 to 26 wherein the step of feeding said food organism to said nematode worm is carried out in liquid culture.

28. A method of inhibiting expression of a target gene in a nematode worm comprising introduction of a DNA capable of producing a double-stranded RNA structure having a nucleotide sequence substantially identical to a portion of said target gene in said nematode, wherein the nematode is one which exhibits increased gut uptake compared to wild type.

29. A method as claimed in claim 28 wherein the nematode is a microscopic nematode.

30. A method as claimed in claim 29 wherein the nematode is from the genus *Caenorhabditis.*

31. A method as claimed in claim 30 wherein the

nematode is *C. elegans*.

32. A method as claimed in any one of claims 28 to 31 wherein the nematode has a mutant genetic background.

33. A method as claimed in claim 32 wherein the nematode is mutant *C. elegans* strain bg85.

34. A method as claimed in any one of claims 28 to 33 wherein the DNA capable of producing a double-stranded RNA structure is a vector comprising a promoter or promoters orientated relative to a DNA sequence such that they are capable of initiating transcription of said DNA sequence to RNA capable of forming a double-stranded structure upon binding of an appropriate RNA polymerase to said promoter or promoters.

35. A method as claimed in claim 34 wherein the vector comprises two promoters flanking the DNA sequence.

36. A method as claimed in claim 35 wherein the two promoters are identical.

37. A method as claimed in claim 34 wherein the vector comprises a single promoter and further comprises said DNA sequence in a sense and an antisense orientation relative to said promoter.

38. A method as claimed in any one of claims 34 to 37 wherein the nematode is adapted to express an RNA polymerase capable of initiating transcription from said promoter or promoters.

39. A method as claimed in any one of claims 34

to 38 wherein the RNA polymerase is T7, T3 or SP6 polymerase.

FIG. 1.

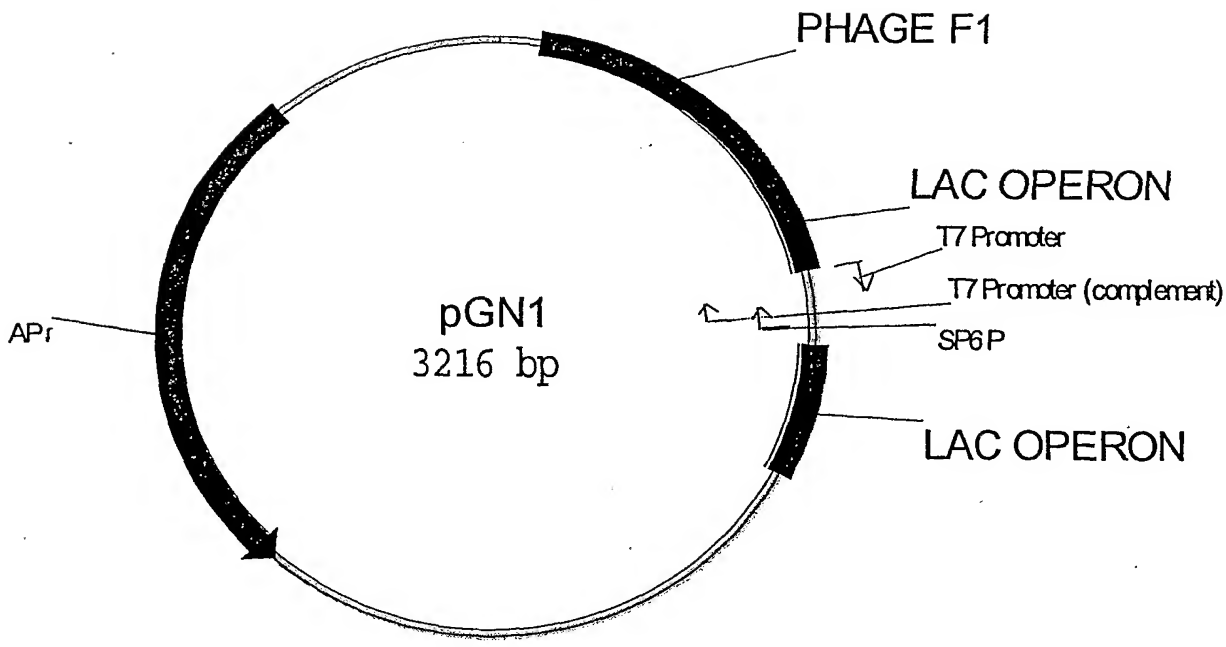

PHAGE F1
LAC OPERON
T7 Promoter
T7 Promoter (complement)
SP6 P
pGN1
3216 bp
APr
LAC OPERON

FIG. 2.

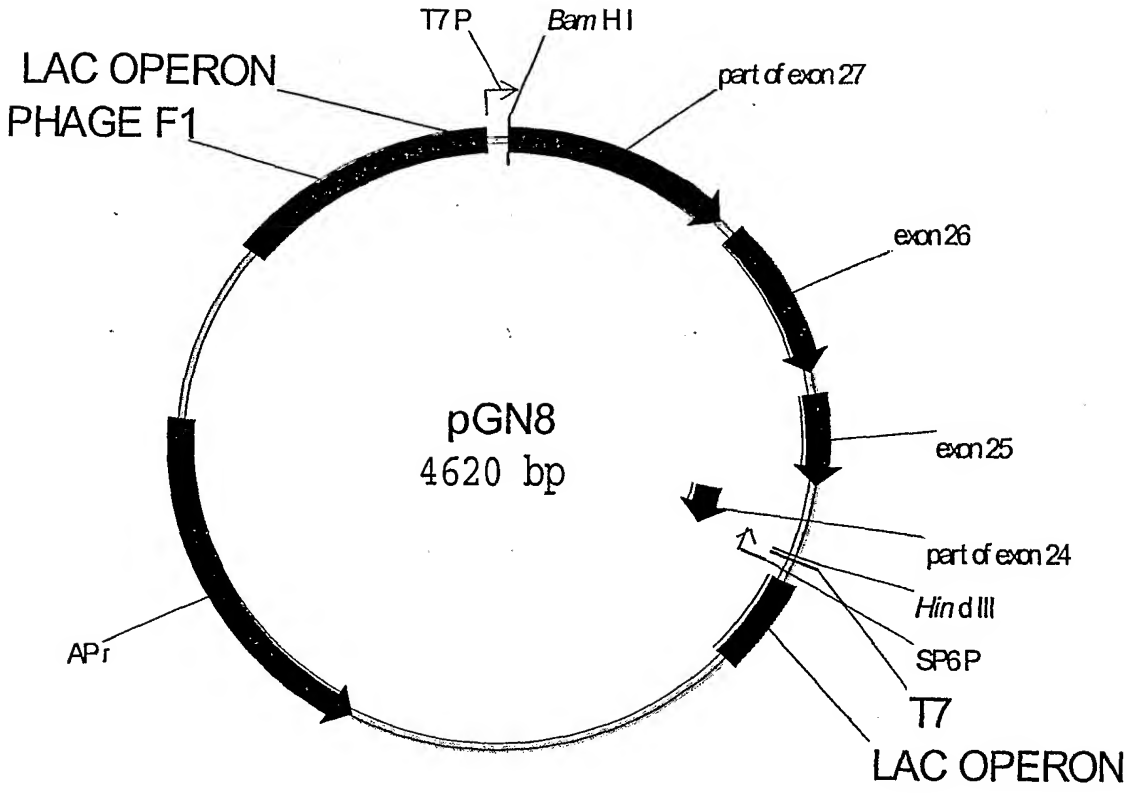

T7 P
Bam HI
LAC OPERON
PHAGE F1
part of exon 27
exon 26
pGN8
4620 bp
exon 25
part of exon 24
Hin d III
SP6 P
T7
LAC OPERON
APr

FIG. 3.

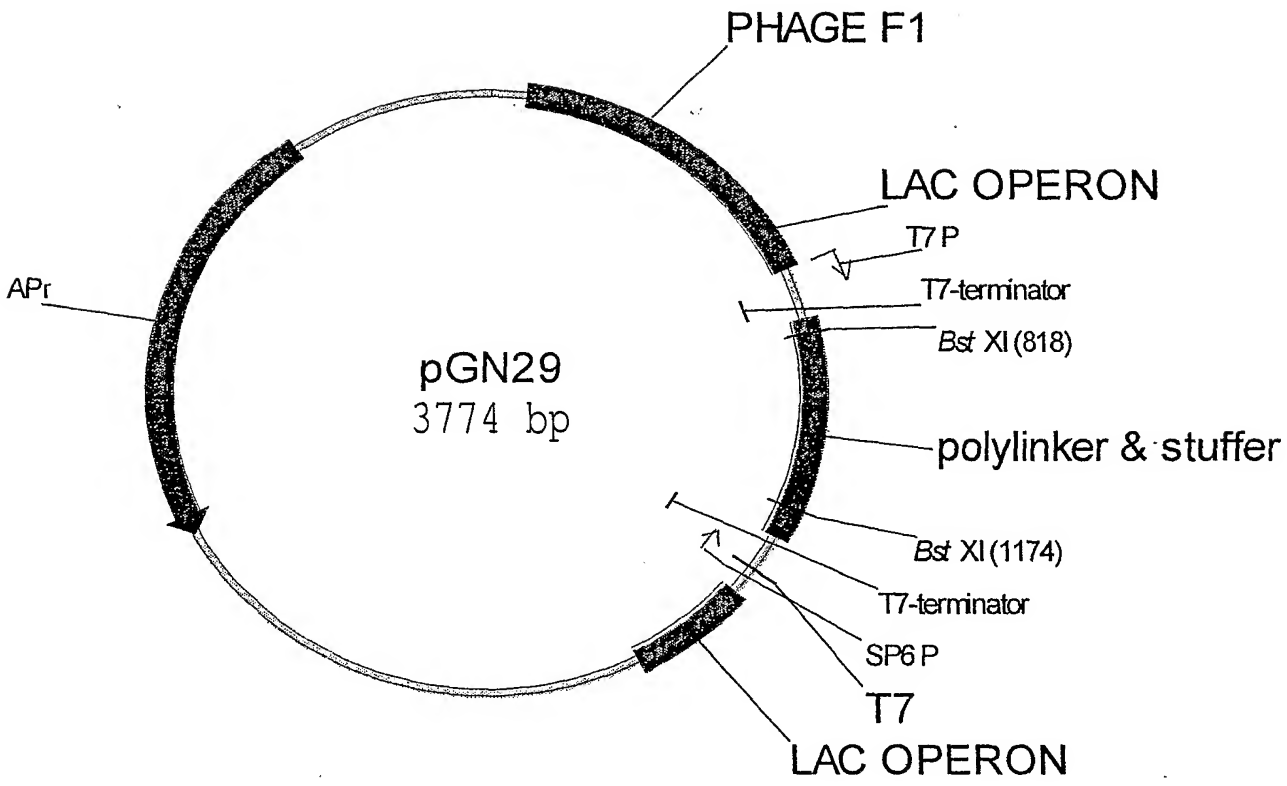

PHAGE F1
LAC OPERON
T7 P
T7-terminator
Bst XI (818)
polylinker & stuffer
Bst XI (1174)
T7-terminator
SP6 P
T7
LAC OPERON
APr
pGN29
3774 bp

FIG. 4.

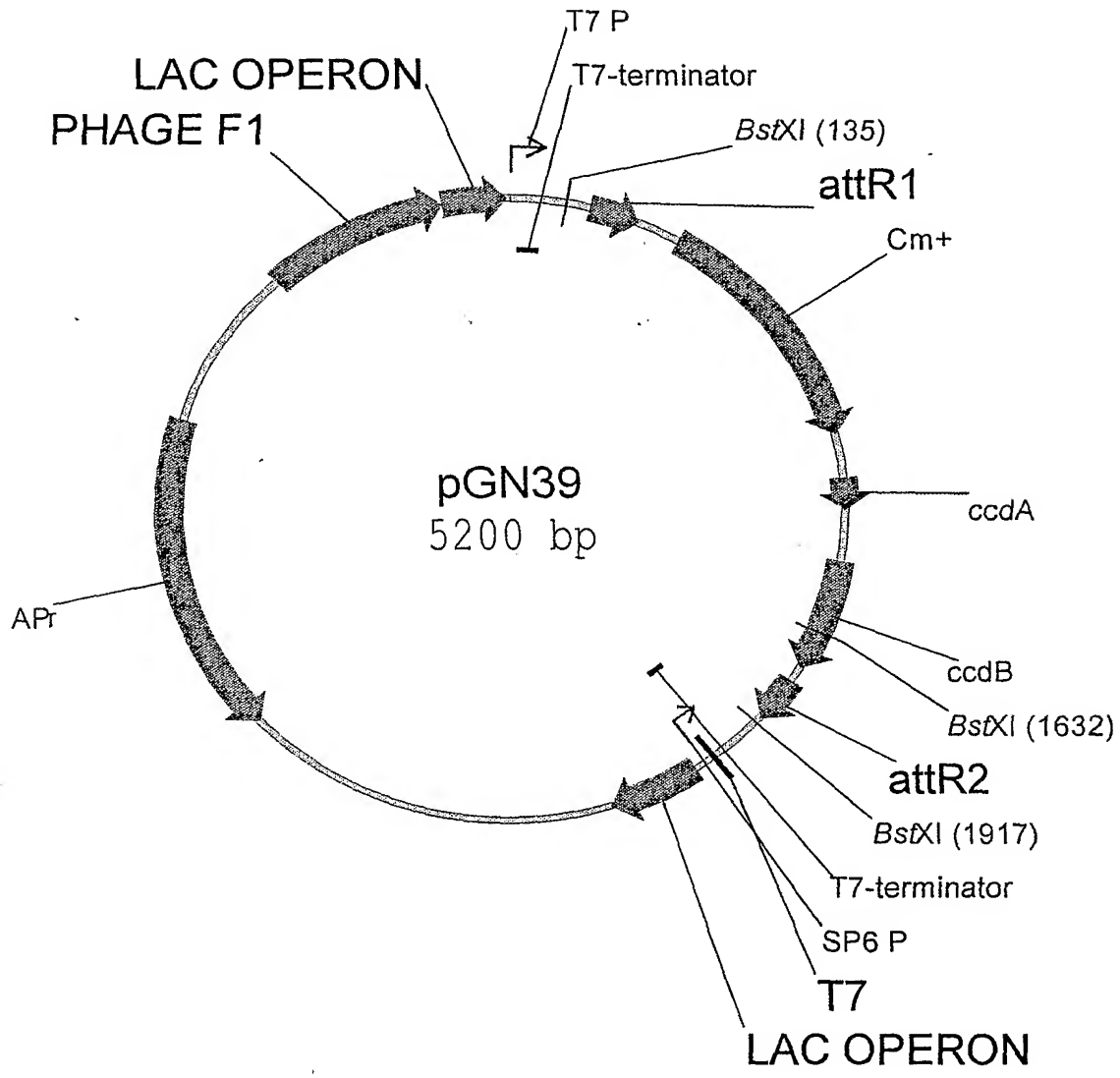

T7 P
T7-terminator
LAC OPERON
PHAGE F1
BstXI (135)
attR1
Cm+
pGN39
5200 bp
ccdA
APr
ccdB
BstXI (1632)
attR2
BstXI (1917)
T7-terminator
SP6 P
T7
LAC OPERON

FIG. 5.

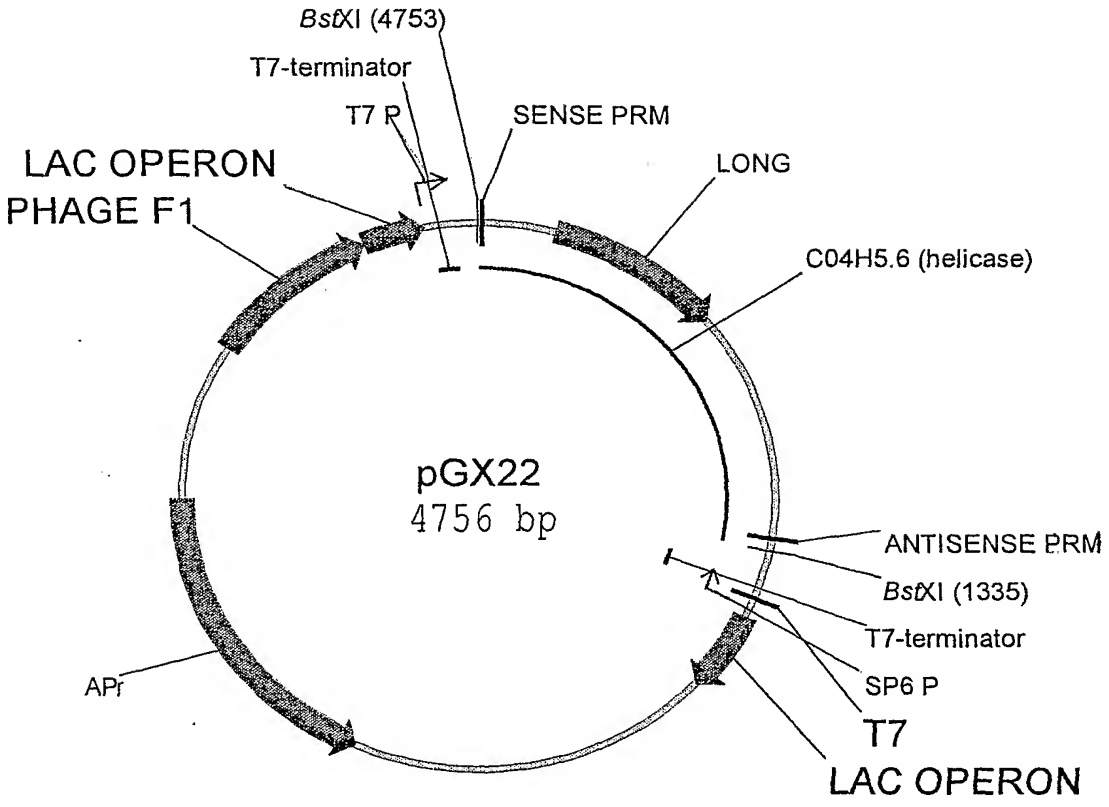

BstXI (4753)
T7-terminator
T7 P
SENSE PRM
LAC OPERON
PHAGE F1
LONG
C04H5.6 (helicase)
pGX22
4756 bp
ANTISENSE PRM
BstXI (1335)
T7-terminator
SP6 P
T7
LAC OPERON
APr

FIG. 6.

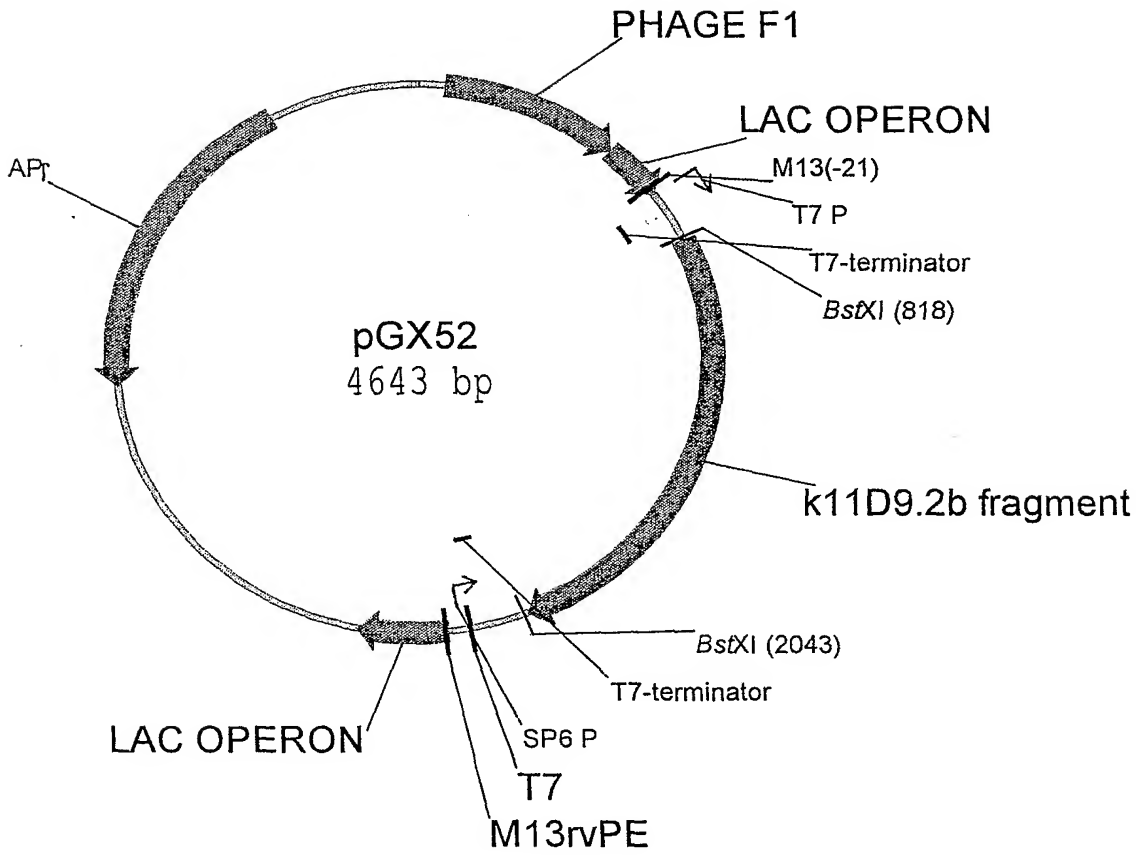

PHAGE F1
LAC OPERON
M13(-21)
T7 P
T7-terminator
BstXI (818)
APr
pGX52
4643 bp
k11D9.2b fragment
BstXI (2043)
T7-terminator
SP6 P
T7
M13rvPE
LAC OPERON

FIG. 7.

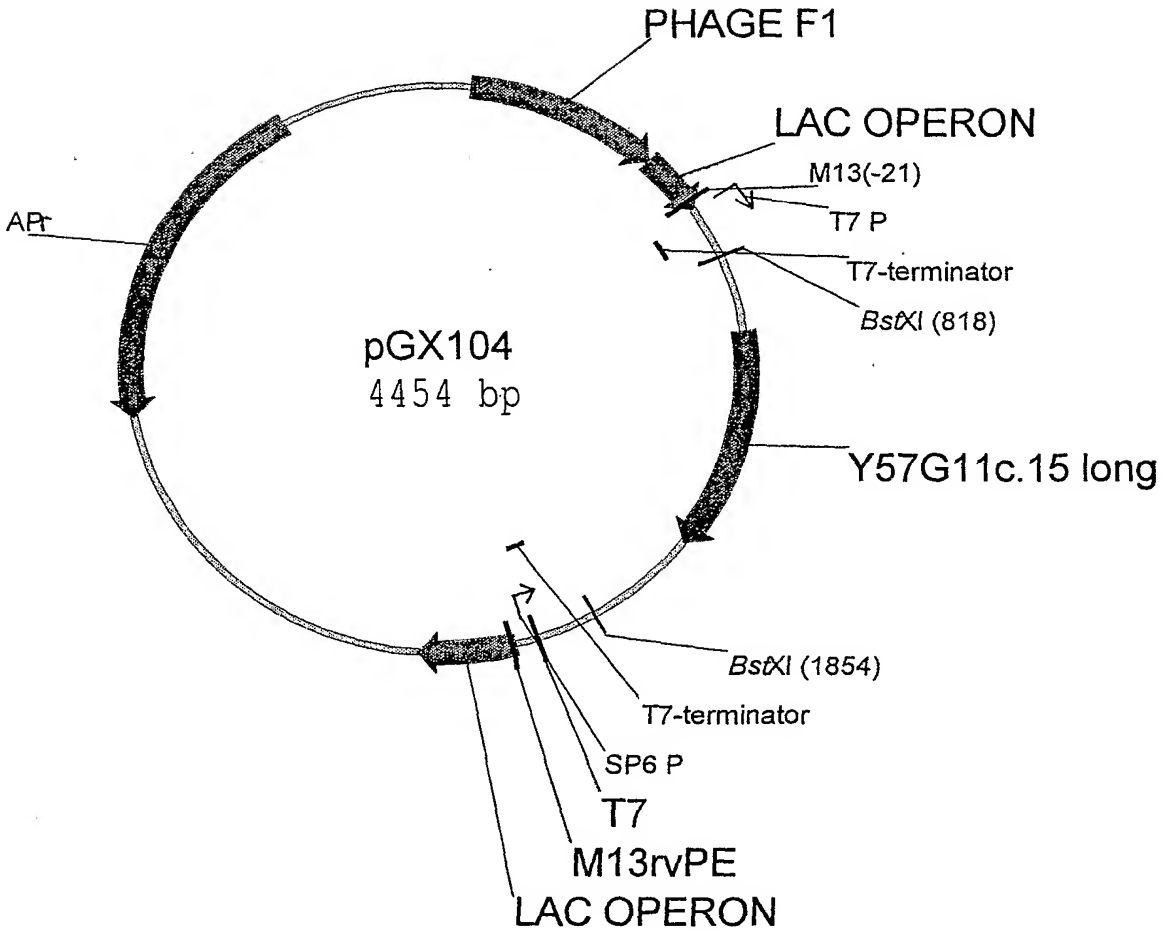

PHAGE F1
LAC OPERON
M13(-21)
T7 P
T7-terminator
BstXI (818)
AP
pGX104
4454 bp
Y57G11c.15 long
BstXI (1854)
T7-terminator
SP6 P
T7
M13rvPE
LAC OPERON

FIG. 8.

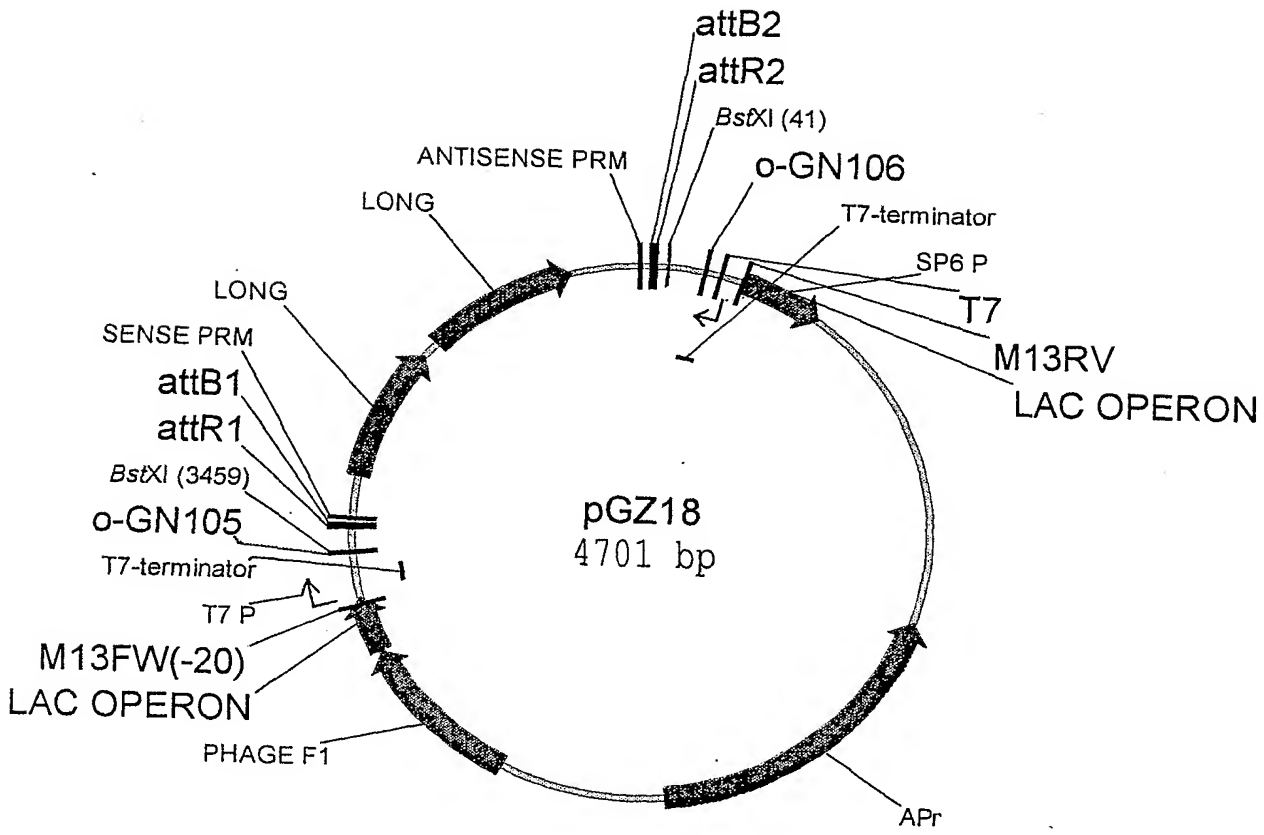

attB2
attR2
BstXI (41)
ANTISENSE PRM
o-GN106
LONG
T7-terminator
SP6 P
LONG
T7
SENSE PRM
M13RV
attB1
LAC OPERON
attR1
BstXI (3459)
pGZ18
4701 bp
o-GN105
T7-terminator
T7 P
M13FW(-20)
LAC OPERON
PHAGE F1
APr

FIG. 9.

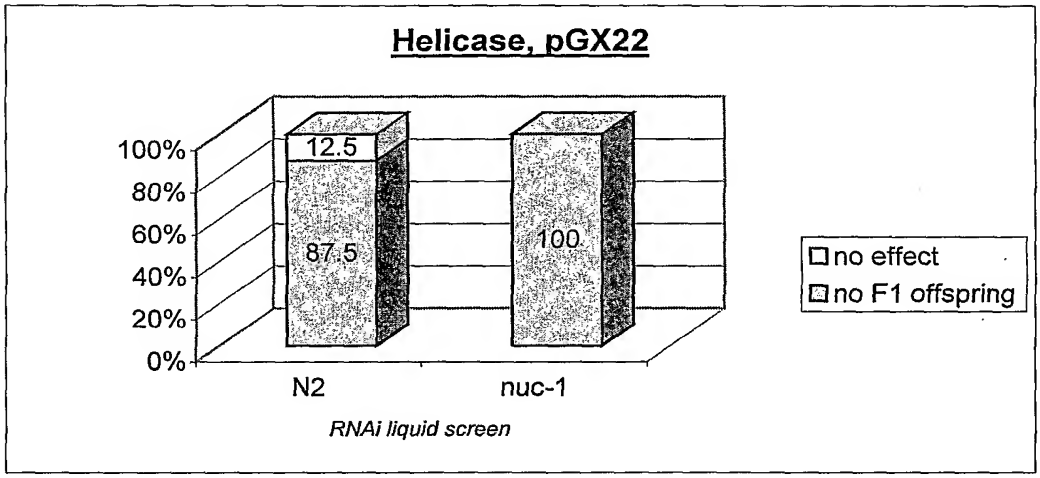

Helicase, pGX22
100%
80%
60%
40%
20%
0%
12.5
87.5
100
N2
nuc-1
RNAi liquid screen
no effect
no F1 offspring

FIG. 10.

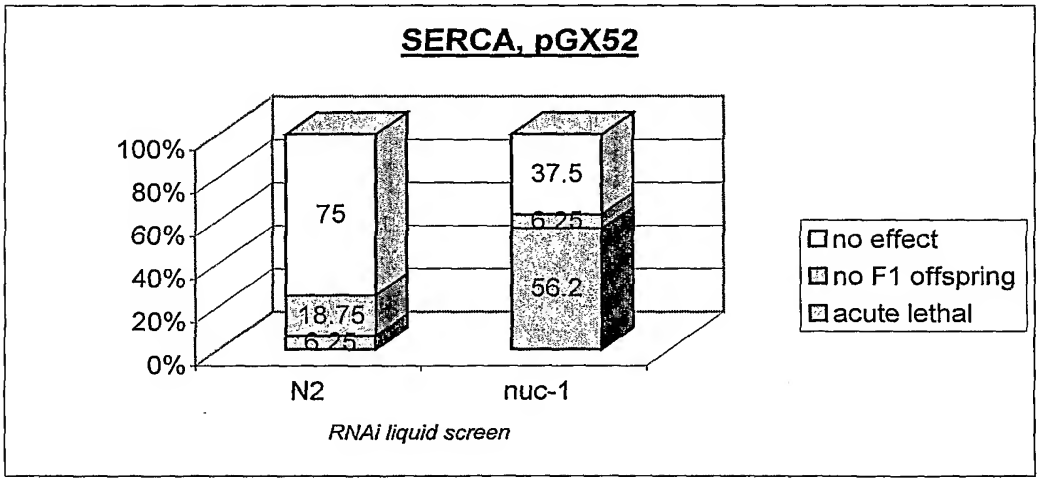
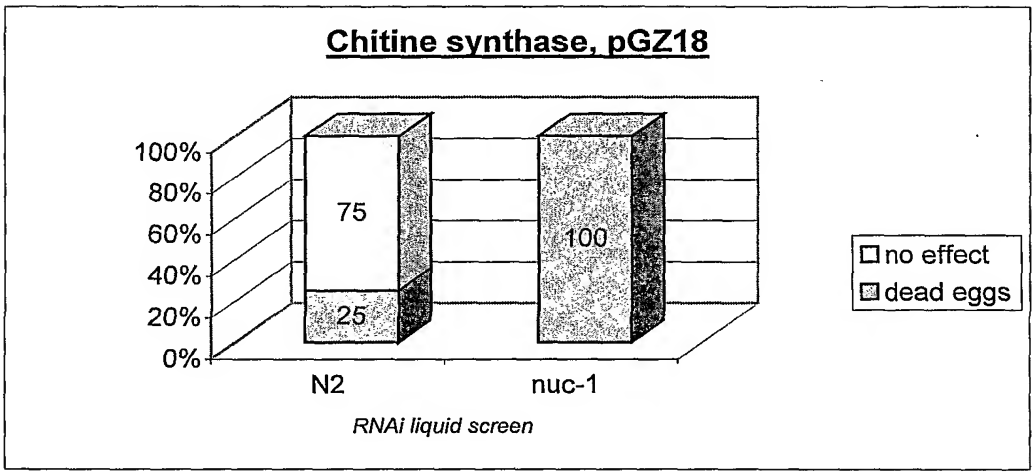

SERCA, pGX52
100%
80%
60%
40%
20%
0%
75
18.75
6.25
37.5
6.25
56.2
N2
nuc-1
RNAi liquid screen
no effect
no F1 offspring
acute lethal

FIG. 11.


Chitine synthase, pGZ18
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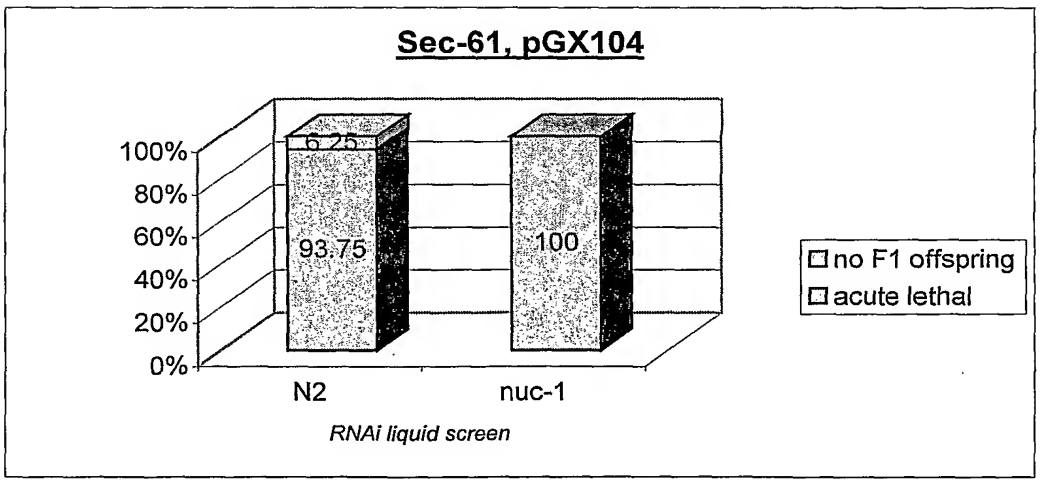
80%
60%
40%
20%
0%
75
25
100
N2
nuc-1
no effect
dead eggs
RNAi liquid screen

FIG. 12.


Sec-61, pGX104
100%
80%
60%
40%
20%
0%
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93.75
100
N2
nuc-1
no F1 offspring
acute lethal
RNAi liquid screen

SEQUENCE LISTING

<110> DEVGEN NV

<120> IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNA INHIBITION

<130> SCB/53711/001

<140>
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<160> 14

<170> PatentIn Ver. 2.0

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<213> Artificial Sequence

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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Plasmid pGN8

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<211> 4643
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<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Plasmid pGX52

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<211> 4454
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Plasmid pGX104

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<213> Artificial Sequence

<220>
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(54) Title: IMPROVEMENTS RELATING TO DOUBLE-STRANDED RNA INHIBITION

(57) Abstract: There are described ways of improving the efficiency of double stranded RNA inhibition as a method of inhibiting gene expression in nematode worms such as *C. elegans*. In particular, the invention relates to the finding that changes in the genetic background of *C. elegans* result in increased sensitivity to double-stranded RNA inhibition.

# INTERNATIONAL SEARCH REPORT

rnational Application No
PCT/EP 00/13149

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C12N9/22 C07K14/435 C12N15/66 C12N15/70
C12N1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal, WPI Data, PAJ

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[X] Further documents are listed in the continuation of box C.

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| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Mateo Rosell, A.M. |

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rnational Application No
PCT/EP 00/13149

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